Supplementary Experimental Procedures

Plasmid construction

We prepared the pDC-attB-var3(HA) plasmid (Supplementary Table S1) in several steps. First, the transgene acceptor cassette from the plasmid pDC (Fidock *et al.*, 2000) was removed using the enzyme SphI, and replaced with an annealed multi-restriction site oligonucleotide linker (made from top strand oligomer 354 and bottom strand oligomer 355; see a listing of this and all others primers in Table S2) to generate the plasmid pDClink (listed along with other plasmids in Table S1). This transfection plasmid expressed the human *dihydrofolate reductase* (h*dhfr*) selectable marker under the control of the 0.6 kb *Plasmodium chabaudi dihydrofolate reductase - thymidylate synthase* (*PcDT*) 5' untranslated region (UTR) and a 0.9 kb *P. berghei DT* 3'UTR. The *P. berghei elongation factor-1alpha* (*ef-1*α) 5' UTR sequence was obtained from a clone kindly provided by Dr. Andy Waters (University of Glasgow, Scotland). The first 900 codons of the *var* gene *IT4var3* were amplified in a two-step reaction, whereby the hemagglutinin (HA) epitope YPYDVPDYA (sequence 5'-TACCCATACGACGTCCCAGACTACGCT) was incorporated between residues 204 and 212 in plasmid pDC-attB-var3(HA), as follows. In the first step of the reaction, a 5' fragment was amplified with the primers SK5 and SK6 and a 3' fragment with SK7 and SK8. These two PCR products overlapped in the HA epitope tag. Overlapping PCR was used to combine these two fragments.

Using the pDC-attB-var3(HA) plasmid, we incorporated the *attB* site (5'-AGGCTTGTCGACGACGGCGGTCTCCGTCGTCAGGATCATCTA) just upstream of the *IT4var3* open reading frame (ORF). By PCR amplification we also created a 5' NotI site and 3' BamHI, SphI, and PstI sites. These amplified fragments were inserted into a pGEM-T plasmid containing the *ef-1*α 5' UTR, and were placed downstream of the promoter using the NotI and PstI cloning sites. The *IT4var3 attB*- and HA-tagged gene elements were inserted into pDClink together with the *ef-1*α promoter in a head-to-head fashion with the h*dhfr* selectable marker cassette using the SphI enzyme.

Plasmids containing the *attP* element were generated from a second *attB*-containing plasmid made concurrently with pDC-attB-var3(HA). This plasmid, termed pDC-var3(attB-HA), was generated as above, except that the

attB element was inserted prior to the HA epitope within the *IT3var3* ORF. To generate the *attP*-containing plasmids, first a BamHI/BglII fragment containing the *attB* site, the HA epitope, and codons 212 to 900 of the *IT4var3* gene was released from the plasmid pDC-var3(attB-HA). This fragment was replaced by an oligonucleotide linker generated by the annealing of primers 2019 and 2020. This linker was engineered to create AvrII and XhoI sites downstream of the *IT4var3* 5' end (encoding amino acids 1-197 and designated "NTS"), with an in-frame V5 epitope (GKPIPNPLLGLDST) contained between these sites. The h*dhfr* cassette was then removed by digestion with NotI plus XbaI, and an oligonucleotide linker (top strand primer 2015, bottom strand primer 2016) was inserted to generate the plasmid p-attP-NTS-V5 (Supplementary Table S1), bearing an SpeI site and a directional *attP* sequence, such that the transgene on the incoming *attP* plasmid would be integrated just downstream of the *ef-1α* promoter in the *attB* locus. The plasmid p-attP-NTS-V5-TM-ATS was generated by the insertion of a 2.3 kb XhoI/BamHI *IT4var3* fragment, consisting of codons 849 to 1324 of the *It4var3* gene and containing the transmembrane (TM) domain and the 3' acidic terminal segment (ATS), together with 0.9 kb of endogenous 3' UTR sequence and the *It4var3* intron. This fragment was PCR amplified from genomic *Plasmodium falciparum* A4 DNA using primers 2021 and 1441 (*Plasmodium falciparum* IT4 genomic sequence information is still incomplete, therefore the sequence of the downstream primer was derived from PFA0015c, the homolog of *IT4var3* in 3D7 parasites). A XhoI-digested GFP fragment was then prepared by PCR amplification from pLN-GFP (Nkrumah *et al.*, 2006), using primers 2023b and 2022 to remove the stop codon and add XhoI sites at either end. This fragment was inserted into the XhoI site of this plasmid to generate p-attP-NTS-V5-GFP-TM-ATS. Plasmid p-attP-NTS-V5-GFP was then created by replacing the NcoI/BamHI fragment containing the TM-ATS 3' UTR region of the transgene with a NcoI/BamHI fragment isolated from pLN-GFP and consisting of the 3' portion of the GFP ORF and the *hsp86* 3' UTR. To generate the plasmid p-attP-NTS-V5-GFP-TM-ATS-*hsp86*, a ClaI/AflII fragment containing the 3' ORF of *IT4var3* was engineered to include an AflII site at the stop codon, by PCR amplification from the p-attP-NTS-V5-GFP-TM-ATS plasmid using primers 1558 and 1557 and subcloning the fragment into pCR2.1 (Invitrogen). This plasmid was opened with AflII plus XbaI and an AflII/XbaI digested fragment containing the *hsp86* 3' UTR (obtained from pLN-GFP) was inserted. Following XbaI and Klenow digestion to create a blunt 3' end, a fragment containing the 3'-ORF and *hsp86* 3' UTR was released by digestion with ClaI. This fragment was ligated into p-attP-NTS-V5-

GFP-TM-ATS prepared in the same fashion but with a blunt-ended BamHI 3' flanking site. The plasmid p-attP-NTS-V5-TM-ATS-GFP was generated from p-attP-NTS-V5-GFP by the insertion of an *IT4var3* 1.6 kb XhoI fragment that was PCR-amplified from p-attP-NTS-V5-GFP-TM-ATS with primers 2021 and 1556r (removing the stop codon to enable the 3' addition of GFP).

Plasmids p-attP-NTS-V5-GFP, p-attP-NTS-V5-GFP-TM-ATS, p-attP-NTS-V5-GFP-TM-ATS-*hsp86*, and p-attP-NTS-V5-TM-ATS-GFP were digested with SpeI to accept a 1.7 kb heterologous *P. falciparum hrp3* promoter. This promoter sequence was generated from pLN-GFP by PCR amplification using primers 1721 and 1722 to introduce 5' AvrII and 3' SpeI sites. Upon ligation into the *attP* vectors, the 5' AvrII site ligated with the 5' SpeI overhanging end, allowing for directional discrimination of inserts and retention of a unique SpeI site. These plasmids are denoted by the term "*hrp3*", such that p-attP-NTS-V5-GFP becomes p-attP-*hrp3*-NTS-V5-GFP, etc. The *attP*-containing plasmids p-attP-*hrp3*-NTS-V5-GFP-TM-ATS-CBD and p-attP-*hrp3*-NTS-V5-TM-ATS-GFP-CBD are depicted in Supplementary Fig. S4A, B.

p-attP-*hrp3*-A4var-V5-TM-ATS-GFP and p-attP-*hrp3*-R29var-V5-TM-ATS-GFP were generated from the plasmid p-attP-*hrp3*-NTS-V5-TM-ATS-GFP by replacement of the NTS fragment with the respective head structures of *var* genes *A4var* or *R29var*, using the enzymes AvrII and SpeI. p-attP-*hrp3*-R29var-V5-GFP-TM-ATS was generated from the plasmid p-attP-*hrp3*-NTS-V5-GFP-TM-ATS-*hsp86* in the same fashion. The *A4var* head structure consisted of the first 846 codons encoding the DBL1α and CIDR1α domains (PCR amplified from A4 genomic DNA using primers SK1 and SK2), while the *R29var* head structure consisted of the first 787 amino acids containing the DBL1α1 and CIDR1α1 domains (PCR amplified from A4 genomic DNA using primers SK3 and SK4).

The drug selectable marker CBD (consisting of a 0.6 kb *calmodulin* 5' UTR driving the *blasticidin S-deaminase* gene with a *P. berghei DT* 3' UTR) was isolated from plasmid pCBD-P (Muhle *et al.*, 2009) by NotI digestion, and ligated into the NotI site of the *attP* plasmids listed above.

CD36 and CSA binding assays

Petri dishes were first coated with 10 µl spots of either 0.1 mg/ml CSA or 0.05 mg/ml CD36-Fc (R&D Systems) overnight at 4˚C. Dishes were then incubated with 2% BSA in 1 × PBS pH 7.4 for 1 hour at 37˚C. The PBS-BSA solution was then removed and 10 µl red blood cells (RBCs) were added on top of the coated spots at a concentration of 10^7 infected RBCs/ml. After 2 hours at 37˚C, we removed non-adherent RBCs by gentle washing with culture medium prewarmed to 37°C. Adherent RBCs were fixed with 1% glutaraldehyde and the numbers of adherent infected RBCs per mm^2 were determined on a Olympus CKX41 microscope at 100 × magnification.

Rosetting assay

To test for rosetting, parasite cultures at 1 % hematocrit were rotated for 15 minutes at 10 rpm/minute at room temperature in the presence of 1 µg/ml ethidium bromide. A drop of cell suspension was then placed on a slide, covered by a coverslip and analyzed with a Nikon Eclipse E600 microscope. Ethidium bromide staining of parasite DNA was used to identify infected RBCs and brightfield images used to determine the presence of bound uninfected RBCs.

Trypsin digestion assay

Infected erythrocytes at the trophozoite stage were incubated for 20 minutes at 37°C with the following concentrations of TPCK-treated trypsin (Sigma) in 1xPBS: 1 mg/ml, 0.1 mg/ml, and 0.01 mg/ml. After trypsinization, cells were incubated with soybean trypsin-chymotrypsin inhibitor (Sigma) for 5 minutes at room temperature and processed for flow cytometry analysis as described in the materials and methods section in the main text.

Electron tomography

Immunolabeled sections of R29-V5-TM-ATS-GFP were prepared as described in the main materials and methods. Tilt series were taken at 11500 × magnification at room temperature with a liquid nitrogen-cooled

Tecnai F20-FEG at 200 keV at a maximal tilt of ±60º. The rotation between two exposures was maximally 2º and followed the Saxton scheme (Saxton *et al.*, 1984). The final tomograms were calculated with the "protomo" software package (Taylor *et al.*, 1999) using marker-free alignment and weighted backprojection.

Supplementary References

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